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Linkage Studies between dark body and ruby eye loci in *Anopheles stephensi*, an urban malaria vector in India

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Abstract
Anopheles stephensi is an important urban malarial vector in the Indian sub-continent which accounts for about 15% of the annual malaria incidence. Chemical control is most effective and immediate in controlling the vectors, but they cause major long term problems such as environmental pollution and resistance. Genetic control is the alternative method of controlling the vectors. In order to develop such a method, it is mandatory that the genetic makeup of the vector species should be established. Linkage study is one such aspect of characterizing the genetic makeup of a vector. Two morphological mutants, dark body (*da)* and ruby eye (*ru)* in *An. stephensi* were isolated and established. These genes were found to be recessive, monofactorial and autosomal. Linkage studies between the two mutants were carried out. Mendelian crosses were made and the results showed that the genes *da* and *ru* are non-linked and could belong to two separate linkage groups. Such studies will aid in the construction of the linkage map and the mutants can be used in basic and applied research.

Keywords: *Anopheles stephensi,* dark body colour, genetic control, linkage map, malaria, mutants, non-linkage, ruby eye colour.

1. Introduction
Mosquitoes belong to the Order Diptera; Family Culicidae. There are about 3,490 species [1]. Not more than 150 species, largely confined to genera *Anopheles, Aedes* and *Culex,* are the indirect cause of morbidity and mortality among humans than any other group of organisms [2]. *Anopheles stephensi* is an important urban malarial vector in the Indian sub-continent which accounts for about 15% of the annual malaria incidence. Chemical control is most effective and immediate in controlling the vectors, but they cause major long-term problems such as environmental pollution and resistance. The insecticides become futile due to the resistance mechanisms in the vectors.
Genetic control is the alternative method of controlling the vectors of disease since it does not cause the problems as conventional control methods. Genetic characterization of mosquitoes, especially of those species and strains which are vectors continue to be an essential component of genetic control strategies aimed at disrupting the transmission of diseases [3]. Such mutant markers could be used in the construction of genetically modified strain/s like genetic sexing systems [4-6]. Many genetic sexing systems were developed by using insecticide resistant genes linking it to the Y chromosome through translocations and along with a visible marker [7-11]. Traditionally, morphological mutants have been used to construct special genetic load strains containing chromosomal translocations or inversions [12-13]. In such strains, genetic markers indicate the presence of the chromosomal aberrations through either altered linkage relationships or position effects of genes located close to chromosomal breakpoints. Genetic markers are necessary for expanding the linkage maps being established for *An. stephensi* [14]. In view of this, it is mandatory that extensive genetic studies of the above said species should be carried out. This paper describes the linkage relationship of two morphological mutants dark body colour (*da*) and ruby eye colour (*ru*) in *An. stephensi.*

2. Materials and methods
Two mutants, whose inheritance pattern has been established, dark body (*da*) [15] and ruby eye (*ru*) [16] were used to test if any linkage exists between these two mutant genes.
Dark larvae (*da*): The body colour of the larvae was dark while compared to the wild type but

the eye colour was that of the wild type (Figure 1). The gene *da* was found to be recessive, monofactorial and autosomal.

Ruby eye (*ru*): The body colour of the larvae was that of the wild type and the eye colour was ruby colour (Figure 1). The gene *ru* was found to be recessive, monofactorial and autosomal.

Dark/Ruby (*da/ru*): A homozygous double mutant with dark body colour and ruby eye was synthesized by crossing both the mutants for two generations. The double mutant was obtained in the second generation by inbreeding F_1 progeny from the parents. This double mutant was reared as a separate stock and was used in the present investigation.

The mutants were maintained like wild type strains. No special care was required to maintain them. These were reared according to the method of Shetty [17]. Colonies of adult mosquitoes were maintained in cages. The cages were made of iron frames covered with mosquito net. Adults were fed with 10% sucrose solution with sterilized cotton soaked in it and adult females were provided with blood meal on restrained mice five days after their emergence. The gravid females lay eggs 48 hrs after taking blood meal. The eggs were kept for 72 hours to ensure complete hatching. The larvae were reared in white enamel pans containing tap water and were fed with powdered yeast tablets on regular schedule throughout the larval period. Stocks were maintained at a relative humidity of 75 ± 5% and temperature at 25 ± 1˚C throughout the course of investigations. The light and dark periods were maintained in the ratio of 10:14 hrs per day.

Cis cross: The cross was conducted between *cis* individual (both the mutant genes in the same individual) i.e. the double mutant and the wild type. The progeny of this cross, heterozygous wild type was crossed with double mutant (*da/ru*) (crosses 1,2,4 and 5) and also was allowed to inbreed to get F_2 generation (cross 3 and 6).

Trans cross; The cross was conducted between trans individuals (both mutant genes in different individuals) i.e. pure bred dark and ruby eye mutants. The progeny of this cross, heterozygous wild type was crossed with double mutant (*da/ru*) (crosses 7,8,10 and 11) and also was allowed to inbreed to get F_2 generation (9 and 12).

Mass matings were made for all genetic crosses. After the blood meal, the females were isolated singly in 35 ml glass vials lined with filter paper and filled with 10 ml water for oviposition. The resulting progeny from each female were reared as an iso-family.

Males and females were separated from freshly emerged adults. Reciprocal, backcrosses and inbreeding of F_1 *inter se* to get F_2 generation were carried out to establish if any linkage exists between the mutants dark and ruby eye of *An. stephensi.* The percentage cross over was calculated for the gene-linkage relationship. The results obtained from all the crosses were subjected to the χ^2 analysis.

3. Results and Discussion

The linkage relationship of dark (*da*) with ruby eye (*ru)* in *An. stephensi* was determined by using classical Mendelian crosses and analysis. A total of 12 crosses (including both *cis* and *trans*) were carried out. Results of these crosses are summarized in Table 1. The F_1 progeny from the individual crosses were backcrossed with double mutant and F_2 progeny were analyzed for the parental and non-parental types.

The results of the crosses summarized showed no linkage between *da* and *ru*. The crosses 1, 2, 3 (*cis* test) and crosses 7, 8 and 9 (*trans* test) involved the F_1 progeny derived from male out cross and similarly, the crosses 4, 5, 6 (*cis* test) and cross 10, 11 and 12 (*trans* test) involved the F_1 progeny derived from female out cross. The results from the crosses 3 and 6 (from *cis* test) and 9 and 12 (from *trans* test) showed a ratio of approximately 9:3:3:1 ratio of wild type, dark, ruby-eye, and double mutant respectively and the expected numbers has been shown in parenthesis for the crosses. *Cis* crosses 3 and 6 showed a ratio of 9.7:1:3.4:3.1 and 9.8:1:3:3.3 while the *trans* crosses 9 and 12 showed a ratio of 2.1:3:10.4:1 and 4.5:4.3:12.6:1 of parental and non-parental types respectively. The results from the crosses 1, 2, 4, 5, 7, 8, 10 and 11 showed nearly 50% cross over to the parental types indicating that the two mutant genes are not linked. χ^2 values were non-significant at $P<0.05$ for all the 12 crosses at 3 degrees of freedom. The two mutant genes segregate independently, hence may be present on different linkage groups (autosomes).

Morphological and larval colour mutants and its genetic basis of inheritance in *An. stephensi* were reported by several pioneer workers. Several workers have reported mutants in *An. stephensi*. The eye colour mutants include X- linked white eye [3], sex linked chestnut eye [18], autosomal colourless eye [19], scarlet, autosomal marron eye [20], pigment-less and red spotted mutant [14], creamish white eye [21], ruby eye [16], sienna eye [22] etc.

Larval colour mutants reported include green [23], yellow [3], brown [24], greyish brown [25], grey [26], greyish black [27], dark [15], green thorax [28], stripe [29], greenish brown [30], black [31], diamond palpus [32], black scale [33], golden-yellow [34], green [35], pale and dark colour [36] etc.

An. stephensi has a diploid chromosome number, 2n = 6. Three pairs of chromosomes (linkage group) are designated as I, II and III based on their length and position of the centromere. It has a pair of sub telocentric sex chromosome (I) and two pairs of autosomes (II and III). The longest pair is designated as group II and the shorter as group III [6, 37]. Linkage studies showed that the ruby-eye locus (*ru*) was non-linked with greyish brown (*grb*) [16] and green thorax (*gt*) locus non-linked with ruby-eye (*ru*) [28]. The present study also showed non-linkage of dark (*da*) with ruby eye (*ru*). Linkage studies between sienna eye (*si*) and *grb* showed that these two genes are closely linked to each other (3.34 ± 1.88 cM) [22].

Golden-yellow (*gy*) and black larva (Bl) mutants were found to be linked with a map distance of 3.75 ± 0.42 and have been placed in linkage group [34]. Linkage analysis showed the sequence of the genes short palpi (sp), diamond palpi (dp), Black larva (Bl) and dieldrin resistance (Dl) as *sp-dp-Bl-Dl* in *An. stephensi* [38]. Spotless wings (sl) and 2nd-3rd costal spots fused (2-3f) have been mapped on chromosome 2, approximately 79.5 map units apart in *An. stephensi* [14]. Three new eye colour mutants, scarlet (wsca), red-spotted (prs) and pigment less (p) were found to be sex-linked in *An. stephensi* [39].

3.1 Tables and Figures

Table 1: Linkage studies between dark and ruby eye mutant larva in *An. stephensi* Liston

Cross no.	Crosses		*Cis* Test						Crossover (%)	χ^2
			Parental type			Non-parental type				
	Male	Female	Wild type	Dark & Ruby eye	Total	Dark	Ruby	Total		
1	da ru / + + Wild Type	da ru / da ru Dark,Rubyeye	94	86	180	81	76	157	46.5	0.14
2	da ru / da ru Dark,Rubyeye	da ru / + + Wild Type	77	61	146	75	72	147	50.1	0.26
3	da ru / + + Wild Type	da ru / + + Wild Type	156 (154.8)	16 (17.2)	172	55 (53)	51 (53)	106	38.1	0.22
4	da ru / + + Wild Type	da ru / da ru Dark,Rubyeye	65	57	122	61	64	125	50.6	0.18
5	da ru / da ru Dark,Rubyeye	da ru / + + Wild Type	77	71	148	76	76	152	50.7	0.24
6	da ru / + + Wild Type	da ru / + + Wild Type	227 (225)	23 (25)	250	77 (73)	69 (73)	146	36.8	0.59
			Trans Test						Crossover (%)	χ^2
			Parental type			Non-Parental type				
7	+ + / da ru Wild Type	da ru / da ru Dark,Rubyeye	Dark 88	Ruby 79	Total 167	Wild type 94	Dark & Ruby eye 89	Total 183	47.7	0.6
8	da ru / da ru Dark,Rubyeye	+ + / da ru Wild Type	79	82	161	85	76	161	50.00	0.54
9	+ + / da ru Wild Type	+ + / da ru Wild Type	32 (38.5)	45 (38.5)	77	157 (154.8)	15 (17.2)	172	30.92	2.24
10	+ + / da ru Wild Type	da ru / da ru Dark,Rubyeye	72	76	148	73	68	141	51.2	0.26
11	da ru / da ru Dark,Rubyeye	+ + / da ru Wild Type	83	82	165	92	77	169	49.4	1.32
12	+ + / da ru Wild Type	+ + / da ru Wild Type	104 (102.5)	101 (102.5)	205	291 (282.6)	23 (31.4)	314	39.49	2.52

df = 3
χ^2 values non-significant at P<0.05
Numbers in parentheses show expected value

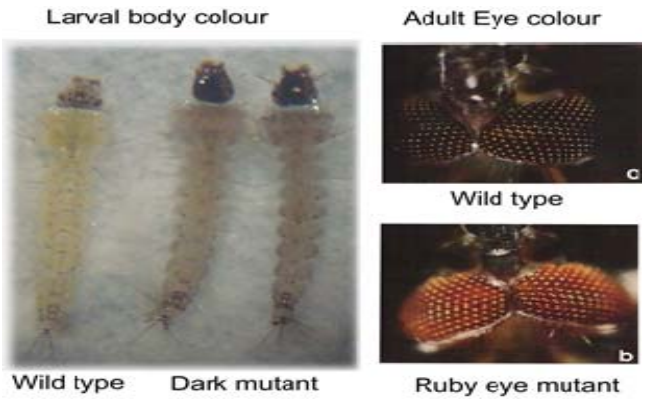

Fig 1: Larval dark body colour and adult ruby eye colour mutants of *Anopheles stephensi.*
(Mutants shown alongside wild type for comparison)

4. Conclusions

The loci for the genes *gt, grb, da* and *si* were found to be non-linked with *ru*. Therefore it could be concluded that all these genes should be on the same chromosome (linkage group). It also indicates that gene *ru* should be present on another linkage group. Linkage studies will aid the construction of linkage maps and can be used in the genetic control methods.

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